# Characterization of *p*-coumarate accumulation, *p*-coumaroyl transferase, and cell wall changes during the development of corn stems

Ronald D Hatfield,* Jane M Marita and Kenneth Frost

*USDA–Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA*

**Abstract**

**BACKGROUND: Developmental changes occur in corn (*Zea mays* L.) stems from cell initiation to fully mature cell types. During cell wall maturation the lignin is acylated with *p*-coumarates (*p*CA). This work describes characterization studies of the *p*-coumaroylation process in relation to corn stem development.**

**RESULTS: Corn plants from three locations were harvested and tissues were analyzed from all nodes and even-numbered internodes above soil line. Changes in carbohydrates reflect a shift to lignification at the expense of structural polysaccharide synthesis. Accumulation of *p*CA paralleled the incorporation of lignin while ferulate (FA) remained relatively constant as a proportion of the cell wall (5–7 g $kg^{-1}$ CW). The *p*-coumaroyl transferase (*p*CAT), which is responsible for attaching *p*CA to lignin monomers, displayed maximum levels of activity in the middle region of the stem (internodes 10–12, 2–3 nmol $L^{-1}$ $min^{-1}$ $mg^{-1}$). The syringyl content as a proportion of the total lignin did not change significantly with cell wall maturation although there was a trend towards increased amounts of syringyl units in the more mature cell walls.**

**CONCLUSIONS: Incorporation of *p*CA into corn cell walls not only mirrored lignification but the *p*CAT activity as well. Levels of *p*CAT activity may be an indicator of rapid lignification specifically for syringyl type lignin. Published in 2008 by John Wiley & Sons, Ltd.**

**Keywords:** Cell wall; *p*-coumarates; *p*-coumaroyl transferase; lignin; corn; acylation

## INTRODUCTION

Several studies have investigated developmental changes in corn (*Zea mays* L.) stem internodes providing detailed changes in cell wall components.[1–3] Particular attention has focused on lignin formation and its impact upon cell wall degradation.[3] Lignins are polymers resulting from dehydrogenative polymerization (via radical coupling reactions) of three primary phenylpropanoid monomers, *p*-coumaryl (H), coniferyl (CA), and sinapyl alcohols (SA).[4] For most land plants, lignins are usually composed entirely of CA (referred to as guaiacyl (G) units) such as found in coniferous gymnosperms or combinations of CA and SA (referred to as guaiacyl (G) and syringyl (S) lignins, respectively) as found in angiosperms. Lignification marks the stage of full cell elongation but not the end of cell wall formation. In many crops, lignins may be acylated by various acids.[5–7] The biochemistry associated with such acylation remains unresolved and the genes involved unknown. Recent work has shown that lignin monomers (monolignols) are acylated first to produce ester conjugates that are then incorporated by coupling and cross-coupling into lignin by the traditional free-radical reactions.[8] Structural studies of corn lignins indicate attachment of *p*-coumarates (*p*CA) primarily on S units over G units (by greater than about 90%) and therefore presumably arises from a preference to *p*CA–sinapyl alcohol (*p*CA–SA) conjugate formation over *p*CA–coniferyl alcohol (*p*CA–CA).[9] The enzyme responsible for this acylation reaction (*p*-coumaroyl transferase, *p*CAT) has been identified and preliminary characterization studies indicate a preference for SA as the alcohol acceptor for the ester conjugate formation (in preparation). The role of this enzyme in the lignification process of grass cell walls remains unclear although it has recently been demonstrated that the formation of *p*CA–SA conjugates may act as radical shuttle mechanisms to aid in the oxidation of sinapyl alcohol residues and subsequent incorporation into lignin polymers.[10]

To help understand the functional role of lignin acylation in corn, a comprehensive structural study of developing stem tissues was carried out using both greenhouse and field grown materials. The objective of

* Correspondence to: Ronald D Hatfield, USDA–Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA
E-mail: ronald.hatfield@ars.usda.gov
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this work was to determine the relationship of *p*CAT activity to other developmental characteristics of cell wall components. A comparison of field to greenhouse-grown corn also has provided information concerning the environmental influence on cell wall development.

## MATERIALS AND METHODS

### Plant materials

Corn inbred B73 (obtained from Dr Jim Coors, Corn Geneticist, University of Wisconsin, Madison, WI) was grown at two field locations, Arlington, WI and West Madison, WI, and in greenhouses at the U.S. Dairy Forage Research Center, Madison, WI. Plots were established in the spring of 2005. Field plots were planted in two 3.7 m rows with 25 cm plant spacing within a row and 90 cm between row spacing. For greenhouse-grown plants, seeds (two per pot) were planted in 2-gallon (approx. 9 L) pots, watered as needed to maintain adequate soil moisture and fertilized once weekly (Pete's Soluble 10-10-10, 5 g $pot^{-1}$ added as water-soluble fertilizer). Plants at the West Madison site were irrigated to prevent drought stress while those at Arlington received no additional water. The months of June (approx. 4.19 cm) and July (approx. 1.19 cm) until harvest on 19 and 20 July were below normal rainfall approx. 10.29 and 9.98 cm respectively for both locations (http://www.wunderground.com). Plants at Arlington without supplemental water were beginning to experience stress conditions, stunted growth and leaf curling during the day. Five plants were harvested from each of three replicated plots at each location. Plants were harvested at the same stage of development – tassel emergence. Plants were cut through the seventh internode (just above the soil line) with leaves and sheaths carefully removed from each stem. Internodes were labeled from 8 to the upper most internode that had undergone elongation (internode 16). The maximum leaf or internode number a plant grows to before switching from a vegetative stage to a reproductive stage can vary between corn varieties and within a corn variety depending on the growing conditions. Because of the dry growing conditions at the Arlington location, plants only reached the 15-internode stage before tassel emergence. Therefore, no data for internode 16 was available at this location. The stem material was cut and separated into nodes, internode lower half, and internode upper half (e.g., node 8 (N8), lower internode 8 (I8B) and upper internode 8 (I8T)). All nodes were processed for analysis, but only the even-number internodes were isolated and processed for analysis. Internode sections were separated into rind and pith portions using a cork bore (size range from 4 to 6) to cut away the pith tissue and only the rind used for further analyses. Initial work indicated that rind tissue contained the higher levels of *p*CAT activity on a fresh weight basis compared to pith tissues and is probably a reflection of more heavily lignified cell walls. Therefore, rind tissues were used in all characterization studies reported here. Similar plant fractions were pooled within replicates at a given location, rapidly homogenized in a Robot Coupe (Model R2UB – 3-quart (approx. 3.42 L) size homogenization chamber fitted with a serrated edged s-shaped homogenization blade) and immediately frozen in liquid nitrogen before storing at −80 °C. Frozen samples were processed for water-soluble phenolics, cell wall carbohydrates, lignin, cell wall phenolics and *p*CAT activity.

### Preparation of stem materials for analysis

Frozen nodes and internodes isolated from corn stems were further processed by grinding in liquid nitrogen using a Waring Blender (300 mL stainless steel vessel) (Torrington, CA). Liquid nitrogen was added to keep the sample frozen. Four 30 s pulses were sufficient to pulverize to a particle size of less than 2 mm. Ground samples were immediately transferred to specimen cups, kept frozen by additional liquid nitrogen, and stored at −80 °C until further processing.

A sub-sample of frozen ground stem material (5 g) was weighed into a 50 mL conical centrifuge tubes. Thawed samples were suspended three times in buffer (50 mmol $L^{-1}$ Tris–acetate, pH 6.7, 2.5 mL $g^{-1}$ sample), sonicated for 10 min, centrifuged at 3200 × *g* for 15 min and the buffer extract carefully removed from the insoluble residue/pellet. The three buffer extracts were combined, frozen in liquid nitrogen and stored at −80 °C until processed for soluble phenolics. Tris buffer (50 mmol $L^{-1}$, pH 6.7) was added to each insoluble residue, and samples were placed in a 90 °C water bath for 2 h. Samples were then transferred to a 55 °C water bath and incubated for 2 h after adding $\alpha$-amylase (Sigma A3403, 10 U $tube^{-1}$) and amyloglucosidase (Fluka 10115, 10 U $tube^{-1}$; St. Louis, MO) for starch removal. Ethyl alcohol (EtOH, 95%) was added to each tube to a final EtOH concentration of 80%. Samples were stirred with a spatula before centrifuging at 3200 × *g* for 15 min. Insoluble residues/pellets recovered from the starch extraction procedure were washed extensively (1 mL solvent $g^{-1}$ fresh tissue). The solvent series included: 80% EtOH (2×), chloroform/methanol 2:1 ($CHCl_3$:MeOH; 1×), and acetone (3×) to remove cytoplasmic contaminants.[11,12] Each wash included 10 min sonication, centrifugation (3200 × *g* for 15 min) and solvent removal. The final retained insoluble residues, isolated cell walls, were air dried under a fume hood before structural analysis.

### *p*-Coumaroyl transferase assay

A second sub-sample of frozen ground stem material was weighed into 2 mL microfuge tubes and suspended in 50 mmol $L^{-1}$ NaOH–MOPS buffer pH 7.5 (2 mL $g^{-1}$ fresh tissue). Samples were kept on ice for 2 h and vortexed every 15 min. Insoluble materials in samples were pelleted by centrifugation (10 min at 13 200 × *g*) and the recovered buffer extracts used



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for the enzyme assay to determine *p*CAT activity according to the method of Marita *et al*.[13] Briefly for each transferase assay, a 0.4 mL aliquot of corn stem extract was added to an 8 mL vial. To each vial, 140 mL of *p*-coumaroyl–coenzymeA (*p*CA–CoA, 2.5 mmol $L^{-1}$), 25 mL of dihydrosinapyl alcohol (DHSA, 20.4 g $L^{-1}$), and 10 mL of catalase (2250 U $mL^{-1}$ to prevent possible oxidation of substrates by peroxidases) were added. Samples were vortexed and incubated at 37 °C for 2.5 h while shaking at 125 rpm. Vials were then placed on ice and 4,4′-ethyldienebisphenol (EDB; Aldrich, St. Louis, MO) added as internal standard (0.025 mL of 2 mg $mL^{-1}$). ENVI-18 SPE columns (Supelco, 1 mL; Supelco, St. Louis, MO) were preconditioned using 2 volumes methanol MeOH followed by 2 volumes acidified water ($dH_2O$) prior to slowly pulling the sample through. Vials were washed twice with approx. 1 mL of $dH_2O$ and added to the column. Reaction products formed by the transferase were eluted from SPE columns with 1 mL of MeOH collected in small test tubes (8 mm × 130 mm). A 0.3 mL aliquot of each sample was evaporated to dryness in reaction vials under air and the remaining sample stored at −20 °C. Assay products (*p*CA–DHSA) were identified and quantified as trimethylsilane derivatives (40 mL TMSI, Pierce (Rockford, IL) and 10 mL pyridine) by GLC-FID on a ZB-5ms column (Zebron; 10 m × 0.1 mm, 0.1 μm film; Torrance, CA). The GLC conditions were injector 315 °C, detector 300 °C, and a temperature program of 220 °C for 1 min, 4 °C $min^{-1}$ to 248 °C, 30 °C $min^{-1}$ to 300 °C and held for 5 min.

## Soluble phenolics

The frozen buffer extracts were thawed and pH adjusted to <2.0 with 2 mol $L^{-1}$ trifluoroacetic acid (TFA). Precipitated materials were pelleted by centrifugation (3200 × *g* for 15 min), and the acidified supernatant was slowly pulled through a C18 solid phase extraction tube (Supelco ENVI-18, 3 mL) under vacuum. All C18 columns were washed with MeOH and equilibrated with acidified $dH_2O$ (pH < 2.0) prior to use. Phenolics bound to the column were washed once with acidified water, the wash discarded and the phenolics eluted form the column using 5 mL of MeOH. Samples were dried in reaction vials and recovered phenolics analyzed as silylated derivatives by GLC-FID. See Cell wall phenolics section for GLC program conditions.

## Structural analysis of corn stems

*Lignin determination*

Isolated cell wall samples (approx. 25 mg) were analyzed for lignin using the acetyl bromide method as modified by Hatfield *et al*.[14] The extinction coefficient used to determine lignin concentration was based on a purified HCl–dioxane lignin isolated from corn stems.[15] Lignin composition was monitored using nitrobenzene oxidation following the procedure of Iiyama and Lam[16] with slight modifications. Briefly 2 mL of 2 mol $L^{-1}$ NaOH and 0.125 mL of nitrobenzene were added to 15–20 mg of cell wall sample in Teflon reaction vials. Vials were sealed and placed inside a second larger reaction vessel (five Telfon vials per large reaction vessel) along with a few milliliters of water. The reaction vessels were heated in a forced air oven (170 °C) for 2 h, removed from the oven and cooled under cold water. Individual Teflon vials were opened and contents transferred to Pyrex culture tubes (15 mm × 250 mm, with Teflon lined caps) with $dH_2O$ (2 × 2 mL). The reaction mixtures were extracted once with chloroform (4 mL), followed by acidification with 12 mol $L^{-1}$ HCl (pH < 2) and extracted with ethyl ether (5 mL, twice) and dichloromethane (5 mL, once). The ether and dicholormethane extracts were combined and 100 mg of EDB (Aldrich, 50 mL of 2 mg $mL^{-1}$ in 95% EtOH (St. Louis, MO)) added as internal standard. One half of the sample volume was evaporated to dryness in reaction vials under filtered air. Nitrobenzene oxidation products were identified and quantified as trimethylsilane derivatives (40 mL TMSI (Pierce) and 10 mL pyridine) using GLC-FID on a ZB-5ms column (Zebron; 30 m × 0.25 mm, 0.25 μm film). The GLC conditions were injector 315 °C, detector 300 °C, and a temperature program of 150 °C for 5 min, 4 °C $min^{-1}$ to 200, 10 °C $min^{-1}$ to 240 °C, 30 °C $min^{-1}$ to 300 °C and hold for 10 min.

*Cell wall phenolics*

Cell walls were analyzed for ester-linked phenolics using the procedure of Grabber *et al*.[17] Phenolics were identified and quantified as trimethylsilane derivatives (40 mL TMSI (Pierce) and 10 mL pyridine) using GLC-FID on a ZB-5ms column (Zebron; 30 m × 0.25 mm, 0.25 μm film). The GLC conditions were injector 315 °C, detector 300 °C, and a temperature program of 220 °C for 1 min, 4 °C $min^{-1}$ to 248, 30 °C $min^{-1}$ to 300 °C and hold for 20 min.

A sample of isolated cell wall was treated with mild acid hydrolysis to determine the ferulate (FA) and *p*CA attachment to arabinosyl side chains of glucuronoarabinoxylans found in corn. Acid labile glycosyl linkage of arabinofuranosyl units is cleaved during hydrolysis, but the ester linkage remains intact and FA–Arabinose (FA–Ara) and *p*CA–Ara can be recovered. Cell walls (approx. 20 mg) were weighed into 4 mL vials and 2 mL of 0.1 mol $L^{-1}$ TFA (Aldrich) added to each. Samples were vortexed prior to incubating in a 100 °C heating block for 1 h. After cooling, samples were filtered through 1 mm glass filters (Acrodiscs; Pall Life Sciences, East Hills, NY). Discs were rinsed twice with $dH_2O$ and collected with the original flow through. ENVI-18 SPE columns (Supelco, 3 mL) were preconditioned with 1 vol $dH_2O$, 1 vol MeOH followed by 1 vol acidified $dH_2O$ (TFA) prior to pulling the sample through. Columns were washed twice with acidified $dH_2O$ and arabinoxylan products were eluted from columns with 100% MeOH. Products were verified as

trimethylsilane derivatives (40 mL TMSI (Pierce) and 10 mL pyridine) using GLC-FID on a ZB-5ms column (Zebron; 30 m × 0.25 mm, 0.25 μm film). The GLC conditions were injector 315 °C, detector 300 °C, and a temperature program of 200 °C for 1 min, 4 °C $min^{-1}$ to 248, 30 °C $min^{-1}$ to 300 °C and hold for 15 min. Peaks were authenticated using model compounds and GLC-mass spectrometry (GLC-MS) using the same column and chromatography conditions.

*Carbohydrate analyses*

Cell wall residues were dried overnight at 55 °C prior to weighing for wall analysis. Samples (approx. 50 mg) were weighed into 15 mL polypropylene conical tubes and hydrolyzed using the Seamen method[18] as modified by Hatfield.[11] Inositol was added (10 mg, 200 mL of 50 mg $mL^{-1}$ solution in water) as internal standard. Filtered sub-samples (4 mL) were dried and sugars converted to alditol acetate derivatives using the procedure of Blakeney *et al*.[19] and analyzed by FID-GLC (Supelco SPB-225 column 30 m × 0.25 mm with 0.25 μm film thickness). Samples removed for total uronosyls were diluted 10-fold with $dH_2O$ and analyzed using the method of Blumekrantz and Asboe-Hansen.[20]

## RESULTS AND DISCUSSION

Corn stems, like all grasses, have unique developmental patterns as the plant changes from vegetative to reproductive stages. All internodes in corn are the pith-filled regions of the stem between nodes (Fig. 1) and are formed by intercalary meristems located at the base of the blade, base of the leaf sheath and the base of the internode on the upper side of a node. Cell elongation occurs in a zone just above the intercalary meristem in a corn stem.[21] Grass plant growth is the result of an increase in the number of cells by cell division in meristematic tissue and by cell enlargement and elongation.[22] It is mainly cell elongation that extends grass stems and leaves to their full height/length at maturity. Within each internode, there is a gradient of cells that range from fully elongated at the top to those that have just been formed by cell division at the bottom, depending upon the age of the internode (Fig. 1). Internodes at the base of the corn stem are fully elongated with little additional change occurring in the cell wall. The nodes of the stem represent complex developmental regions, forming a junction between internodes that differ in cell wall formation (fully expanded cells below and recently formed cells above). Nodes also are the attachment sites of leaf sheaths that cover and protect the internode directly above the node of attachment.

The main focus of this work was to understand the relationship of *p*CAT and *p*CA incorporation into corn cell walls and wall lignification along the length of its stem. Detailed analysis of cell wall characteristics was necessary to clearly understand the overall relationship of *p*CAT and *p*CA incorporation to key developmental characteristics in the corn stem and to relate this work to previous developmental studies involving corn. Only the rind tissues of corn stems were chosen for detailed analyses. Previous work indicated similar developmental patterns for the major cell wall components (e.g., lignin, carbohydrates, phenolics) when comparing pith and/or rind tissues of corn stems (mg $g^{-1}$ CW).[1,3] The results are discussed as similar trends of corn stem development across locations, much of the data is presented as means of field replicates for individual locations rather than cumulative means. Differences in overall plant development at tassel emergence (e.g., 15 nodes/internodes (Arlington) vs 17 nodes/internodes (Madison West, greenhouse)) because of diverse growing conditions resulted in different phases of physiological development for identical node/internode numbers among plants from the three different locations.

Wall-bound *p*CA is incorporated as part of the lignin fraction. This incorporation is dependent upon the activity of *p*CAT primarily forming *p*CA–SA conjugates in the cytoplasm that are subsequently deposited into the wall matrix during cell wall lignification (manuscript in preparation). The *p*CAT utilizes *p*CA–CoA as the activated donor molecule and primarily SA as the acceptor molecule. A robust assay, based on GLC-MS, was developed that allows rapid assessment of transferase activity in tissue extracts and authentication of correct product formation. Specific *p*CAT activities (Fig. 2) were determined for all corn stem tissue samples and parallel what is known about lignification within internodes in developing corn stems.[1] If lignification is active, it might be expected

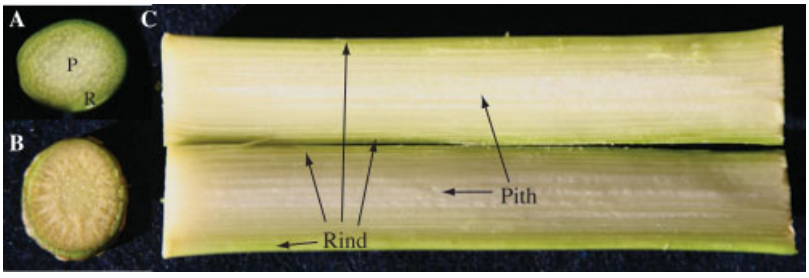


**Figure 1.** Pictorial representation of corn stem internode 8 and node 8 sections. (A) Cross-section through a node/internode interface (R, rind; P, pith); (B) cross section through a lower node showing developmental complexity; (C) longitudinal section through an internode showing the soft pith region and the fibrous rind tissues.



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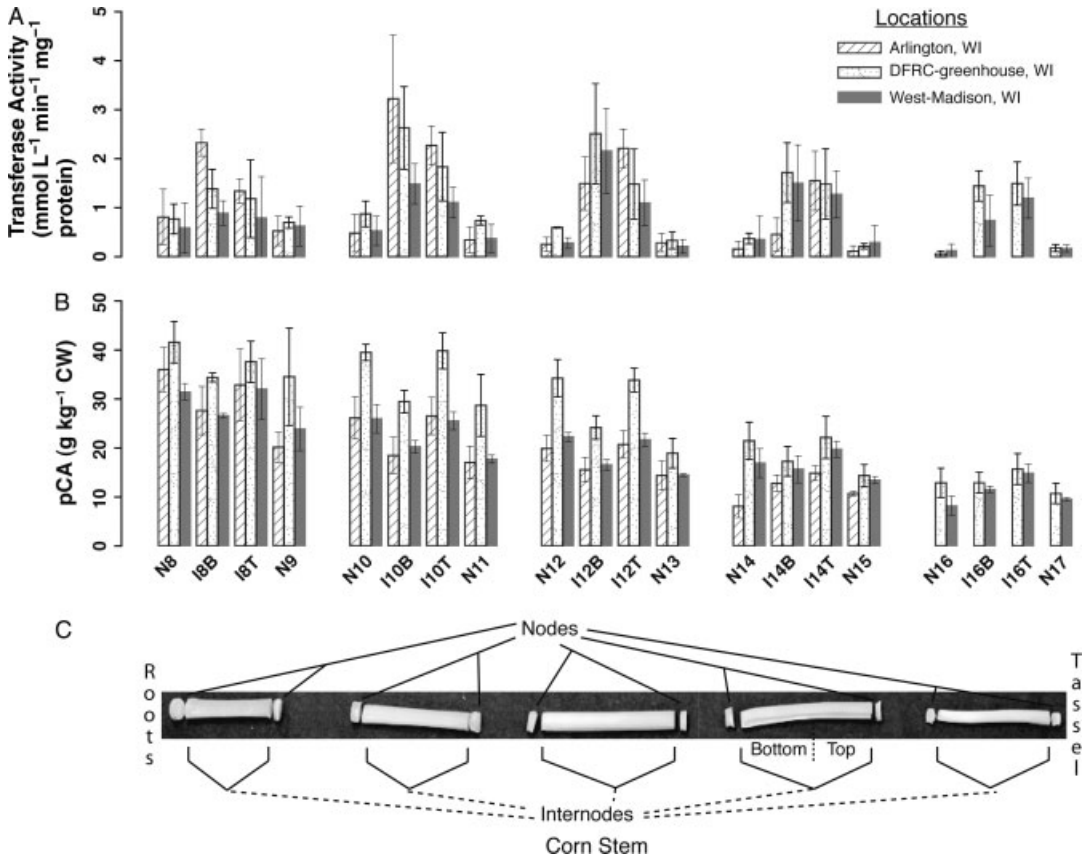


**Figure 2.** Developmental profile of (A) *p*-coumaroyl transferase activity and (B) *p*-coumarates in rind tissues of corn stems. Results are the mean of three location replicates. (C) Pictorial representation of nodes and internodes tissues used for analyses. N, node;, IB, internode bottom half; IT, internode top half.

that the transferase are equally active. In the most mature stem cells, the lower nodes and internodes where lignification is declining, low transferase activity is detected. Similarly, in the still actively elongating tissue in the uppermost internodes where lignification has recently begun, there is low transferase activity. However, in the middle internodes (Fig. 2) where rapid lignification is occurring the *p*CAT activity is highest.

Incorporated *p*CA is most prominent in the older tissues during latter stages of secondary cell wall formation with nodes and internodes showing the same decreasing pattern of incorporation from stem base to tip. It is thought that at latter stages, lignification is ongoing but shifts to incorporate higher amounts of syringyl (S) units into the lignin polymer.[1,23] In this study, the S components are a significant part of lignin even within the most immature internodes as determined by nitrobenzene oxidation. There was a range in values of S for the different locations. A comparison of the most immature sections West Madison I16B (0.080 mol kg$^{-1}$ lig), Arlington I14B (0.050 mol kg$^{-1}$ lig), greenhouse I16B (0.165 mol kg$^{-1}$ lig) to the most mature sections at each location West Madison I8T (0.080 mol kg$^{-1}$ lig), Arlington I8T (0.183 mol kg$^{-1}$ lig), greenhouse I8T (0.10 mol kg$^{-1}$ lig) indicated differences between the locations but little change in the proportion of S in total lignin during stem development. Nitrobenzene oxidation gives a relative composition of lignin by releasing lignin subunits that are ether linked within the polymer. Subunits linked through other types of bonds are not effectively released nor accounted for by this method or by any other known method for degrading lignin. Therefore, changes in S and G units as measured by nitrobenzene oxidation are indicative of the changes in the relative amounts within the releasable lignin subunits. There are measurable amounts of S units throughout all nodes/internodes examined indicating lignin produced in corn rind cell walls is enriched in S units even at early stages of development. Since the primary conjugate that is formed by the *p*CAT appears to be *p*CA–SA, this would fit the observed patterns of *p*CA incorporation into immature stem tissues of corn (Fig. 2). There is not a one-to-one correspondence between levels of *p*CA in the wall and the level of SA incorporated into lignin. Evaluation of tissues for buffer soluble phenolics indicated there were no large pools of soluble ferulate (FA) or *p*CA (data not shown). This would suggest there is a relatively tight coupling of production of these acids and their utilization in formation of cell wall structural components. Additional work is needed to fully understand the flux of monolignols into the wall matrix, incorporation into lignin and critical aspects of that control of lignification including using methods that would allow determination of the total S, G, and

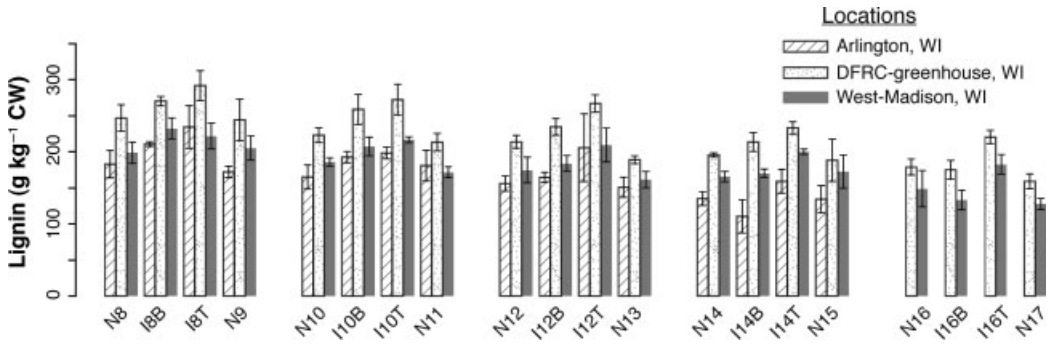


**Figure 3.** Lignin distribution along corn stems as measured by the acetyl bromide method. Individual symbols represent nodes and adjacent internodes tissues. N, nodes; IB, internode bottom half; IT, internode top half. Results are the mean of three location replicates.

H levels within the lignin not just what can be released by oxidative analysis.

Observed acetyl bromide lignin levels (Fig. 3) and ester-linked *p*CA levels within nodes and internodes (Fig. 2) support a mechanism of *p*CA incorporation that mirrors lignin formation. Typically, the lignin content of node cell walls is less than the lignin content of the cell walls in either the upper or lower sections of the surrounding internode tissues (Fig. 3). Levels of esterified *p*CA in the cell walls of the nodes showed a different pattern of incorporation than within internodes. Higher levels of esterified *p*CA were found in a given node than in adjacent internode sections (e.g., node 10 compared to internode 10B or any internode beneath it). This pattern generally persisted up the corn stem. Although the lignin content of nodes was not equivalent to internode sections along the corn stem, lignin composition along the corn stem was similar. The amount of lignin and esterified *p*CA (g $kg^{-1}$ CW)) is greatest in the lower nodes/internodes (8–9) with the uppermost nodes/internodes (16–17) having the least amounts. In general, the top of a given internode has more *p*CA incorporated than the lower half. Again, this mirrors the level and higher degree of lignification in the more mature tissues/cells.

FA is a phenolic closely related to *p*CA. Incorporation of FA into the cell wall occurs as an ester-linked residue on arabinose (Ara) branch units of glucuronoarabinoxylans (GAXs).[24] Morrison *et al*.[1] previously showed that FA incorporation into internode rind and pith tissues of corn stems remains relatively constant across all internodes. The present study concurs with previous work and shows that FA is continually incorporated into secondary cell walls (typical of the vascular xylem and fiber cells in the corn rind) as well as primary cell walls. It has been shown that FA becomes coupled to lignin during the lignification process[25] through ether and carbon–carbon type linkages and is an important part of secondary wall formation. Ferulates coupled to lignin would not have been released during the alkaline treatments employed in these studies. The near constant levels of ester-linked FA measured across the internode/node tissues of the developing corn stems in this study are indicative of their continued incorporation regardless of the node or internode analyzed along the stem (6.0 g $kg^{-1}$ CW). There appears to be no relationship between FA and *p*CA incorporation in the wall matrix.

Grasses can also incorporate small amounts of *p*CA as esterified units on Ara branches of GAXs into wall matrices.[26] To potentially explain the higher levels of esterified *p*CA found in the node tissues, a mild acid hydrolysis was performed on isolated cell walls. The goal was to determine if nodes tissues contained significant amounts of *p*CA bound to GAX. Mild acid hydrolysis cleaves the highly acid labile arabinofuranosyl glycosyl linkage at the point of attachment to the main xylosyl backbone. The ester linkage (FA–Ara or *p*CA–Ara) remains intact and FA–Ara and *p*CA–Ara are recovered and quantified by GLC-FID. No detectable levels of *p*CA–Ara were released from the cell walls treated by mild acid hydrolysis. Significant amounts of FA–Ara were recovered indicating that *p*CA incorporation into walls is predominately with lignin and not as a carbohydrate component (data not shown). The reason why there is greater incorporation of *p*CA into the cell walls of nodes (relative to lignin content), unlike within internodes, will be examined more closely in future studies.

The profile of cell wall carbohydrates changes during internode development and is similar to previous studies.[1,3] The complexity of individual sugar distribution along corn stems can be simplified by looking at the data in general terms across all tissues. One observation is that carbohydrates of nodes have a much more consistent pattern of distribution then the internodes. It is not clear why this would be the case, but is most likely due in part to the complexity of these tissues.

Glucose (Glc) is primarily derived from cellulose deposition in the cell wall, however during primary wall formation, there can also be significant amounts of mixed-linked $\beta$-glucans and limited xyloglucans.[27] Mixed-linked $\beta$-glucans are degraded from the wall during rapid cell elongation, therefore depending upon the distribution and degree of maturation of cells in each section, there may be levels of Glc that should not be assigned strictly to cellulose. Figure 4 shows the distribution pattern of Glc along the corn stem. The highest Glc content is in the middle nodes/internodes (10–12) and in the bottom



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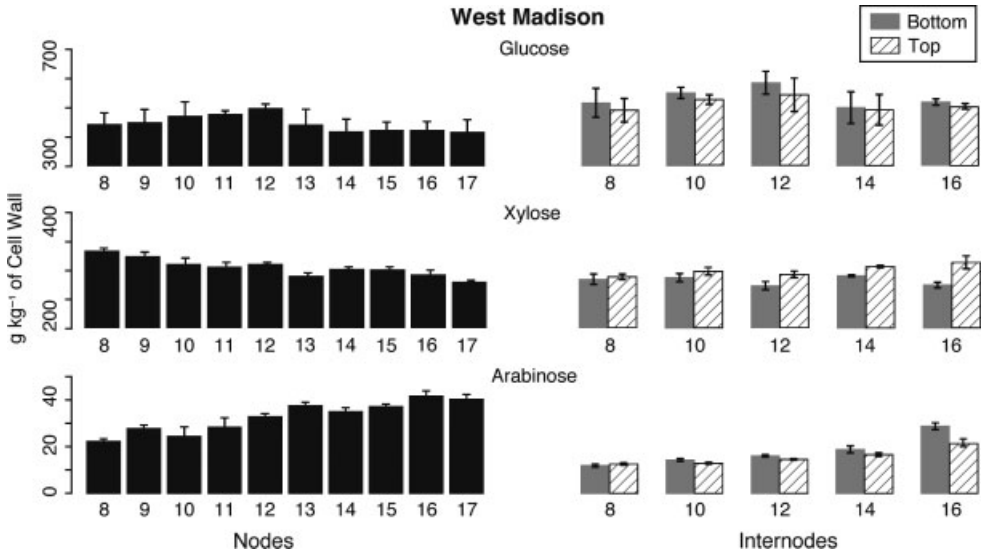


**Figure 4.** Glucose, xylose and arabinose represent the major neutral sugars within corn rind tissue cell walls. Results for all nodes and even numbered internodes (gray for the bottom half of the internode and cross-hatched as the top half of the internode) as the mean of three location replicates. Results are shown for only one location as all locations followed the same developmental patterns for cell wall carbohydrates.

half of the rind tissues within a given internode. These nodes/internodes represent tissues that are accumulating cellulose as a predominate portion of the total cell wall matrix. In regions where there are significant changes in secondary wall formation (new carbohydrate deposition), there would be higher amounts of Glc on a cell wall basis. As the wall continues to mature, lignification increases and carbohydrate deposition decreases. This would result in higher proportional amounts of Glc in less lignified sections and lower proportions in the more mature heavily lignified regions. The node tissues show a similar pattern of Glc deposition within cell walls with nodes 10 through 12 showing the highest amounts.

Xylose (Xyl) the second most abundant cell wall neutral sugar is primarily deposited into corn cell walls as GAX. Although there is variation in the amount of Xyl measured within the upper and lower halves of the internodes along the corn stem, these differences are not significant (Fig. 4). In the case of node tissues, there is a steady decline in Xyl content with decreased maturity (lower nodes to upper nodes). There appears to be a relationship between Xyl and lignin content at least within the node tissues, i.e., the more mature the node the higher the lignin content and Xyl. The cell wall distribution of Ara tends to have higher concentrations within cell walls of less mature tissues (Fig. 4; i.e., bottom sections of the upper most internodes and the upper most nodes). In corn, developing cell walls contain xylans that are highly substituted with Ara, and more mature cell walls contain xylans with lower arabinosyl substitution.[28] In primary cell walls of corn, xylans are synthesized highly substituted with Ara residues that are later hydrolyzed off the main chain to produce a more rigid cell wall.[28,29] It is not clear if highly Ara-substituted xylans are produced during secondary wall formation and modified to contain less Ara substitution prior to being incorporated into the wall matrix. Node tissues show a stronger shift towards higher amounts of Ara in cell walls isolated from immature tissues.

In general, the patterns of the minor cell wall sugars (galactose, mannose, fucose, rhamnose, and uronosyls) either remain constant or show slight decreases as cell walls mature (comparing bottom internodes to upper internodes, e.g., 9 vs 14 and comparing the upper half of a given internode that is most mature with the lower half that is less mature). The one exception is galactose with similar patterns of deposition as Ara whereby higher concentrations (12–15 g $kg^{-1}$ CW) are formed in cell walls of least mature tissues (nodes and lower internode sections, data not shown). It has been shown that arabinogalactans are incorporated into primary walls of corn and experience significant turnover as these walls continue to develop.[29] It is not possible to tell from this data if there is a turnover of galactose in corn rind cell walls or whether it is simply a dilution by other sugars incorporated during secondary cell wall formation. The observed patterns of decreasing sugar concentrations with stem development may indicate synthesis of specific polysaccharides during primary cell wall formation that is discontinued or significantly reduced upon initiation of secondary cell wall formation.

As stated earlier, results are presented and discussed as means of field replicates for each location. All locations followed the same developmental trends, but not all plants were synchronized in terms of peak activities coupled with specific internodes. Included is a statistical analysis of major cell wall components for each location. Nodes and internodes were separated out for analyses because of their inherent differences. Significance tables, based on ANOVA, for individual locations examining nodes and internodes with respect to major parameters of

interest are presented in Table 1. Both lignin and *p*CA had significant interactions ($P < 0.001$) with both nodes and internodes regardless of location emphasizing the similarity in trends across locations. It is not be surprising there were inconsistencies in significant interactions for *p*CAT as there are several variables (e.g., time of harvest, point of lignification, enzyme turnover, extraction efficiency) that impact total enzyme activity extracted from the individual tissues in corn stems. Trends in activity observed suggest that high *p*CAT activity may be related to regions of most active lignification. More detailed information is needed concerning rates of lignin formation, incorporation of *p*CA and *p*CAT activity in order to draw conclusions. There are significant interactions between lignin with respect to nodes or internodes along the stem. Cell wall incorporation of *p*CA follows the same pattern as lignin across all three locations while Ara follows the opposite trend within all locations, i.e., as the stem matures Ara in the wall declines.

All statistical analyses were performed using R ver. 2.4 (http://www.r-project.org/). First, linear models were fit to all data sets, and then the linear model fits were used to compute analysis of variance tables (ANOVA). Three $\alpha$ levels representing three increasing levels of significance are presented in Table 1. Results from minor sugars (galactose, mannose, rhamnose and fucose) and uronic acids were not included because such small measurable amounts were present.

**Table 1.** Table of significance, based on ANOVA, for individual locations examining nodes and internodes with respect to major developmental parameters investigated in this study

| Location and parameter | Nodes (N) $N_{num}$ | Internodes (I) $I_{num}$ |
|---|---|---|
| Arlington | | |
| Lignin | *** | *** |
| *p*CAT | – | ** |
| *p*CA | *** | *** |
| FA | – | ** |
| Glucose | – | * |
| Xylose | – | – |
| Arabinose | * | *** |
| Greenhouse | | |
| Lignin | *** | *** |
| *p*CAT | *** | – |
| *p*CA | *** | *** |
| FA | – | * |
| Glucose | – | – |
| Xylose | – | *** |
| Arabinose | *** | * |
| West Madison | | |
| Lignin | *** | *** |
| *p*CAT | – | – |
| *p*CA | *** | *** |
| FA | – | *** |
| Glucose | – | – |
| Xylose | *** | – |
| Arabinose | *** | *** |

Significance levels:
*** $P < 0.001$;
** $P < 0.01$;
* $P < 0.05$.
*p*CA, *p*-courmarates; *p*CAT, *p*-courmaroyl transferase; FA, ferulates.

## CONCLUSIONS

There is a strong relationship between lignification and *p*-coumaroylation of lignin monomers during corn stem development. Based on the presence of *p*CAT activity and *p*CA in the corn rind cell walls, incorporation of *p*CA begins early in cell wall development and continues as long as lignin monomers are being synthesized and polymerized in the cell wall. SA is the primary acceptor molecule for *p*CAT suggesting *p*CAT has a role in the proper formation of S rich lignins. The mild acid hydrolysis data supports the conclusion that *p*CA is attached to lignin (not Ara) as the major form of incorporation into the cell wall.

The development of corn stems is a multifaceted process involving multiple tissue/cell types. Nodes of corn plants are unique and add even more complexity to stem development. They are transition zones between well-developed cells on the lower adjoining internode to less mature cells on the upper adjoining internode. These stem characteristics are the result of the unique developmental pattern in most grasses whereby both apical and intercalary meristems are present. Synthesis of structural polysaccharides is dominated by cellulose and GAX, although it would appear that small amounts of other structural polysaccharides are also continually produced and incorporated into the wall matrix.

It appears that *p*CA plays a key role in the formation of corn stem lignins that are S rich and that grass lignin is highly *p*-coumaroylated. Since lignin negatively influences corn stem digestibility, the regulation of *p*CAT may provide an avenue for altering lignin composition/concentration in corn and other grasses that would lead to increased digestibility or energy conversion efficiency for utilization as ruminant feeds or bioenergy production from biomass. This approach is currently being explored.

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## REFERENCES


1 Morrison TA, Jung HG, Buxton DR and Hatfield RD, Cell-wall composition of maize internodes of varying maturity. *Crop Sci* **38**:455–460 (1998).

2 Jung HG, Maize stem tissues: ferulate deposition in developing internode cell walls. *Phytochemistry* **63**:543–549 (2003).

3 Scobbie L, Russell W, Provan GJ and Chesson A, The newly extended maize internode: A model for the study of secondary cell wall formation and consequences for digestibility. *J Sci Food Agric* **61**:217–225 (1993).

4 Freudenberg K and Neish AC, *Constitution and Biosynthesis of Lignin*. Springer-Verlag, Berlin (1968).

5 Smith DCC, Ester groups in lignin. *Nature* **176**:267–268 (1955).

6 Harris PJ and Hartley RD, Phenolic constituents of the cell walls of monocotyledons. *Biochem Syst Ecol* **8**:153–160 (1980).

7 Hartley RD and Harris PJ, Phenolic constituents of the cell walls of dicotyledons. *Biochem Syst Ecol* **9**:189–203 (1981).

8 Lu F and Ralph J, Preliminary evidence for sinapyl acetate as a lignin monomer in kenaf. *Chem Commun* 90–91 (2002). [accessed July 25 2008].

9 Grabber JH, Quideau S and Ralph J, *p*-Coumaroylated syringyl units in maize lignin; implications for $\beta$-ether cleavage by thioacidolysis. *Phytochemistry* **43**:1189–1194 (1996).

10 Hatfield R, JR and Grabber JH, A potential role for sinapyl *p*-coumarate as a radical transfer mechanism in grass lignin formation. *Planta* **XX**:XX–XX (2008).

11 Hatfield RD, Carbohydrate composition of alfalfa cell walls isolated from stem sections differing maturity. *J Agric Food Chem* **40**:424–430 (1992).

12 Hatfield RD, Wilson JR and Mertens DR, Composition of cell walls isolated from cell types of grain sorghum stems. *J Sci Food Agric* **79**:891–899 (1999).

13 Marita JM, Grabber J, Lu F, Kim H, Krantz F, Ralph J, *et al.*, Development of a specific assay for *p*-coumaroylation of lignin monomers. *Plant Methods* submitted (2008).

14 Hatfield RD, Grabber JH, Ralph J and Brei K, Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: some cautionary notes. *J Agric Food Chem* **47**:628–632 (1999).

15 Fukushima RS and Hatfield RD, Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. *J Agric Food Chem* **49**:3133–3139 (2001).

16 Iiyama K and Lam TBT, Lignin in wheat internodes. Part 1: the reactivities of lignin units during alkaline nitrobenzene oxidation. *J Sci Food Agric* **51**:481–491 (1990).

17 Grabber JH, Hatfield RD, Ralph J, Zon J and Amrhein N, Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochemistry* **40**:1077–1082 (1995).

18 Saeman JF, Moore WE and Millett MA, Sugar units present. Hydrolysis and quantitative paper chromatography, in *Cellulose*, ed. by Whistler RL. Academic Press, New York, pp. 54–69 (1963).

19 Blakeney AB, Harris PJ, Henry RJ, Stone BA and Norris T, Gas-chromatography of alditol acetates on a high-polarity bonded-phase vitreous-silica column. *J Chromatogr* **249**:180–182 (1982).

20 Blumenkrantz N and Asboe-Hansen G, New method for quantitative determination of uronic acids. *Anal Biochem* **54**:484–489 (1973).

21 Esau K, *Anatomy of Seed Plants*. John Wiley, Chichester (1977).

22 Dahl BE, Developmental morphology of plants, in *Wildland Plants: Phisiological Ecology and Developmental Morphology*, ed. by Bedunah DJ and Sosebee RE. Society for Range Management, Denver, pp. 22–58 (1995).

23 Terashima N, Fukushima K, He L-F and Takabe K, Comprehensive model of the lignified plant cell wall, in *Forage Cell Wall Structure and Digestibility*, ed. by Jung HG, Buxton DR, Hatfield RD and Ralph J. ASA-CSSA-SSSA, Madison, pp. 247–270 (1993).

24 Ishii T and Hiroi T, Isolation and characterization of feruloylated arabinoxylan oligosaccharides from bamboo shoot cell walls. *Carbohyd Res* **196**:175–183 (1990).

25 Ralph J, Grabber JH and Hatfield RD, Lignin–ferulate crosslinks in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohyd Res* **275**:167–178 (1995).

26 Mueller-Harvey I, Hartley RD, Harris PJ and Curzon EH, Linkage of *p*-coumaryl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohyd Res* **148**:71–85 (1986).

27 Carpita N, Hemicellulosic polymers of cell walls of *Zea* coleoptiles. *Plant Physiol* **72**:515–521 (1983).

28 Carpita NC, Cell wall development in maize coleoptiles. *Plant Physiol* **76**:205–212 (1984).

29 Gibeaut DM and Carpita NC, Tracing cell wall biogenesis in intact cells and plants. *Plant Physiol* **97**:551–561 (1991).